PTO 03-5286

CY=JA DATE=19830730 KIND=A
PN=58-128322

CARCINOSTATIC AGENT
[SEIGANZAI]

Kunihiro Shinpo, et al.

UNITED STATES PATENT AND TRADEMARK OFFICE
Washington, D.C. September 2003

Translated by: FLS, Inc.

PUBLICATION COUNTRY	(19):	JP
DOCUMENT NUMBER	(11):	58128322
DOCUMENT KIND	(12):	A
PUBLICATION DATE	(43):	19830730
PUBLICATION DATE	(45):	
APPLICATION NUMBER	(21):	57011116
APPLICATION DATE	(22):	19820127
ADDITION TO	(61):	
INTERNATIONAL CLASSIFICATION	(51):	
DOMESTIC CLASSIFICATION	(52):	A61K 35/80; //A61K 37/02
PRIORITY COUNTRY	(33):	
PRIORITY NUMBER	(31):	
PRIORITY DATE	(32):	
INVENTOR	(72):	SHINPO; KUNIHIRO, ET AL.
APPLICANT	(71):	CHLORELLA INDUSTRY CO., LTD.
TITLE	(54):	CARCINOSTATIC AGENT
FOREIGN TITLE	[54A]:	TOMEI CHAKUSHOKU TSURIITO

Specifications

1. Title of the Invention

Carcinostatic Agent

2. Claim(s)

A carcinostatic agent which is a glycoprotein isolated from green microalgae, such as chlorella, scenedesmus, and spirulina, and has characteristics including a molecular weight of 121,000, isoelectric point (pH) of 8.6, glycoprotein ratio of about 1:1, and protein helix content of about 19%.

3. Detailed Specifications

The present invention relates to a carcinostatic agent derived from green microalgae, such as chlorella, scenedesmus, and spirulina.

Conventional glycoprotein-based antitumor drugs have a host intervening (immunoactivation activity) function, so they exhibit antitumor properties *in vivo* but no direct cytocidal action *in vitro*.

Thus, as a result of investigating the presence of carcinostatic activity on certain kinds of constituents contained in aforesaid algae in the course of researching green microalgae, such as chlorella, and painstaking research on such constituents based on the investigation, an inventor of the present invention discovered that a glycoprotein constituent having a specific molecular weight, isoelectric point, glycoprotein ratio, and protein helix content exhibited antitumor activity both *in vivo* and *in vitro*, specifically acted only on tumor cells, and manifested far superior carcinostatic activity without affecting normal

cells. In short, the carcinostatic agent of the present invention is an epoch-making antitumor drug for solving the non-selectivity of chemotherapeutic agents, which has been a major problem to this day, without affecting normal cells despite showing direct action on tumor cells.

That is, the present invention is a glycoprotein isolated from green microalgae, such as chlorella, scenedesmus, and spirulina, and has characteristics including a molecular weight of 121,000, isoelectric point (pH) of 8.6, glycoprotein ratio of about 1:1, and protein helix content of about 19%.

A practical example of a method in which the carcinostatic agent of the present invention is isolated from chlorella is described next.

A raw chlorella cake cultivated with carbon dioxide, acetic acid, glucose, or the like as the carbon source, or chlorella algal bodies (powder) obtained by blow-drying or freeze-drying this raw cake are prepared as the raw material. 500 g of chlorella algal bodies (5 L in the case of raw chlorella cake) was dispersed in 5 L of water, subjected to a 95 to 100°C hot water extraction for 20 to 30 minutes, subsequently centrifugally separated for 20 to 30 minutes at 3,000 to 10,000 rpm, and the supernatant thereof was obtained as a chlorella hot water extract. The medium used in above extraction is not limited to hot water; there are no impediments to using water with a weak acid or alkali.

About 75 g of hot water extract (based on powder) were obtained from 500 g of chlorella algal bodies by this operation. The hot water extract was concentrated under reduced pressure at 50°C, and the total volume

was brought to 1 L by adding distilled water.

This was passed through a semipermeable membrane and fractionated into a high molecular weight fraction (called fraction **A**, hereafter), which did not pass through the semipermeable membrane, and a low molecular weight fraction (called fraction **B**, hereafter). The yields of **A** and **B** were 45 g and 30 g, respectively (both weights based on powder). The resultant fraction **A** was further concentrated to 500 mL under reduced pressure, after which it was added to a DEAE-cellulose column and subjected to a multistep elution while changing the buffer at each step.

That is, the fraction eluted with M/50 carbonate buffer was designated $\mathbf{A}_1$, the fraction eluted with M-50 carbonate buffer containing M/10 sodium chloride was designated $\mathbf{A}_2$, and the fraction eluted with M/50 carbonate buffer containing 1M sodium chloride was designated $\mathbf{A}_3$, respectively.

The yields of $\mathbf{A}_1$, $\mathbf{A}_2$, and $\mathbf{A}_3$ were 13.0 g, 5.5 g, and 10 g, respectively (all weights based on powder).

The fraction $\mathbf{A}_1$ was concentrated under reduced pressure at 50°C, desalinated using a dialysis membrane, subsequently passed through a Sephadex G-150 column, fractionation was performed by using M/15 phosphate buffer (pH: 7.17), and the resultant fraction was dialyzed and freeze-dried. The yield was 140 mg (about 0.025 % of chlorella algal bodies).

The characteristics of the substance obtained in the above-mentioned extraction method will now be listed.

(i) Molecular weight

ⓐ Molecular weight (calculated) by electrophoresis: 128,000

ⓑ Molecular weight using analytic ultracentrifuge (made by Hitachi Ltd.; 282-0060 type absorption scanning recorder): 121,000

The [average] molecular weight from above-mentioned ⓐ and ⓑ was 121,000.

(ii) Purity

It was proven that the obtained glycoprotein was pure by:

ⓐ gel filtration

ⓑ the sedimentation pattern in an analytical untracentrifugation, and

ⓒ electrophoresis in SDS polycrylamide gel.

(iii) Isoelectric point

pH: 8.6

(iv) Constitutive sugars

The resultant substance was hydrolyzed for 3 hours in 100°C in hydrochloric acid, isolated by thin paper chromatography and cellulose column chromatography, and the moiety and quantity of sugar were analyzed in an orcinol-sulfuric acid method. The results thereof are shown in Table 1 below.

Table 1

Constitutive Sugar	Amount (%)
Galactose	16.4
Glucose	25.5
Mannose	17.6
Rhamnose	14.0
Ribose	20.3
Xylose	6.2

(v) Constitutive amino acid residues

The moiety and quantity of the amino acids were analyzed using an automatic amino acid analyzer (made by Hitachi Ltd.; KLA-3B type). The results thereof are shown in Table 2.

Table 2

Constitute Amino Acid Residue	Amount (%)	Constitute Amino Acid Residue	Amount (%)
Lysine	6.6	Alanine	10.5
Histidine	2.3	Cystine	-
Arginine	4.2	Valine	7.5
Aspartic Acid	13.2	Methionine	0.1
Threonine	6.2	Isoleucine	4.2
Serine	6.0	Leucine	8.4
Glutamic Acid	12.6	Tyrosine	4.2
Proline	4.5	Phenylalanine	4.0
Glycine	5.5		

(vi) Sugar/protein moiety ratio

490 μg/mg:510 μg/mg (about 1:1)

(vii) Infrared spectroscopic analysis

The crystal state was examined using an infrared spectroscopic analyzer (made by Hitachi, Ltd.; 260-10 type) ($^{IR}\ r^{kBr}_{max}\cdot cm^{-1}$)

As a result, absorption peaks belonging to α helix amide (I), (II) and (III) bands were confirmed at 1,640, 1,550, and 1,260 nm, and those of random coil structures were confirmed at 1,660, 1,530 and 1,240 nm.

Moreover, an equatorial H bending vibration peak at the C_1 position of α-glucan position was confirmed at 870 nm, a pyranose ring breathing vibration was observed at 880 nm, an axial H bending vibration at the C_1 position of β-glucan was observed at 906 nm, a C-O-C asymmetrical bending vibration of these pyranose rings was observed at 910 and 920 nm, and the characteristic absorption peak of β-glucan was confirmed at 999 nm,

respectively.

(viii) Argon laser-Raman spectroscopic analysis

The structure was examined in a solution state using an argon laser Raman spectroscopic analyzer (made by JEOL Ltd.; JRS-V1-UV) ($\gamma_{max}^{H_2O(D_2O)}$ cm^{-1}).

As a result, an α-helix amide (I) band was confirmed at 1,642 nm and an α-helix amide (I) band was confirmed at 1,309 nm (symmetrical E_2 species), 1,294 nm (symmetrical E_1 species), and 1,275 nm (symmetrical A species), respectively. The latter band was verified because it disappeared due to conversion of deuterium from deuterium oxide.

Moreover, an absorption peak belonging to an amide (I) band having a random coil structure was observed at 1,663 nm and to an amide (II) band having a random coil structure which disappeared according to deuterium conversion) at 1,257 nm, respectively.

Furthermore, if the respective symmetrical species **A** or $\mathbf{E_1}$ overlap, the bending mode of the amide (IV) band was seen at one 556 nm or 528 nm absorption peak and 379 nm, and the breathing vibration of the ring having a relatively large molecular weight and an α-helix structure due to a hydrophobic amino acid was seen at 350 nm.

The α-helix structures of the substances obtained in above-mentioned (vii) and (viii) are maintained in a crystal state or solution state.

(ix) Circular dichroism spectrum (CD curve)

The α-helix content was examined using an optical rotatory dispersion spectrophotometer (made by Hitachi, Ltd.; UV-5 type) (CD solution of pH 7.0).

As a result, a crossover point on the CD curve was seen at 200 nm, and a double minimum at [θ]-9500 was seen at 209 nm and one at [θ]-7590 was seen at 222 nm. Thus, the α-helix content was 18.9% (about 19%).

The main hydrophobic amino acid group corresponds to substantially 35% of the amino acid residues according to the constitutive amino acid residue, and the sugar moiety per molecule was considered to be substantially equivalent to 20%. If the α-helix structure was mainly constituted of a hydrophobic amino acid, α-helix is considered to be localized to one or several sites on the molecular chain.

The carcinostatic activity of the aforementioned resultant substances was verified by the following tests.

Practical Example 1 (*in vitro* test)

(1-1) Measurement in floating culture method

A medium was implanted with cells inoculated with a cultivated strain of murine lymphocytic leukemia (L-1210/V/C) so that the number of cells were 5×10^4 cells/mp. Moreover, the pH of this medium was adjusted to 7.0 after adding 100 μg/mL of streptomycin and 100 unit/mL of penicillin to RPMI 1640 (GIBCO) containing 10% FCS.

Next, the above-mentioned medium was applied to a suspension culture method for 72 hours at 37°C, and each group to which a fixed amount of this substance was added and a control group without it added were prepared. Upon comparatively judging the number of cells after cultivating these groups, whereas the non-additive control group showed a 100% reproductive rate, the IC-50 (50 % growth-inhibiting concentration) was 2 μg/mL in

the group to which this substance was added.

(1-2) Measurement in dry agar cultivation method

The growth-inhibiting concentration IC-50 of L-1210/V/C of this substance according to the usual method (soft agar cloning analysis) was determined to be 2 μg/mL.

Practical Example 2 (*in vivo* test)

(2-1) Test using Sarcoma 180

10^6 of Sarcoma 180 were intraperitoneally transplanted into one group of thirty 5-week-old ddd mice, then 10 mg/kg of this substance were administered intraperitoneally into mice continuously for five days once per day twenty-four hours after transplantation, and the ratio of the number of days the mice survived with respect to mice not administered with this substance was examined. As a result, the ratio of surviving days (T/C) was 180 %.

(2-2) Test using P-388

10^6 of leucocytes of mice suffering from lymphocytic leukemia (P-388) were transplanted intraperitoneally into one group of thirty 5-week-old CDF_1 mice, then 10 mg/kg of this substance were administered intraperitoneally once per day to mice continuously for five days twenty-four hours after the transplantation, and the ratio of the number of days the mice survived with respect to mice not administered with this substance was examined in the usual method. As a result, the ratio of surviving days (T/C) was 160 %.

(2-3) Test using L-1210

10^5 of leucocytes from mice suffering from lymphocytic leukemia (L-1210) were transplanted intraperitoneally into one group thirty 5-week-old CDF_1 mice, then 10 mg/kg of this substance was administered intraperitoneally once per day to mice continuously for five days twenty-four hours after the transplantation, and the number of days the mice survived with respect to mice not administered with this substance was examined in the usual method. As a result, the ratio of surviving days (T/C) was 145 %.

Practical Example 3 (Immunological test, etc.)

Upon administering a rabbit with 33 mg/kg of this substance through a vein in the ear and another 33 mg/kg of it ten days after that, no anaphylactic findings were confirmed.

Moreover, when this substance was set aside for one day and a rabbit was administered with 5 mg/kg of it a total of six times, no distinct sedimentation line for a normal rabbit was confirmed by immunological electrophoresis even when during administration at the aforesaid conditions.

Thus, it is seen that the antigenicity of this substance is low, that is, its antibody titer is also low. Moreover, this substance was not confirmed either with hemolytic coagulation reactions.